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Crystallization and preliminary crystallographic studies of a new crystal form of *Escherichia coli* L-asparaginase II (Ser58Ala mutant)

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Periplasmic *Escherichia coli* L-asparaginase II with an Ser58Ala mutation in the active-site cavity has been crystallized in a new orthorhombic form (space group $P2_12_12$). Crystals of this polymorph suitable for X-ray diffraction have been obtained by vapour diffusion using two sets of conditions: (i) 1% agarose gel using MPD as precipitant (pH 4.8) and (ii) liquid droplets using PEG-MME 550 (pH 9.0). The crystals grown in agarose gel are characterized by unit-cell parameters a = 226.9, b = 128.4, c = 61.9 Å and diffract to 2.3 Å resolution. The asymmetric unit contains six protein molecules arranged into one pseudo-222-symmetric homotetramer and an active-site competent dimer from which another homotetramer is generated by crystallographic symmetry.

1. Introduction

L-Asparaginases catalyze the hydrolysis of L-asparagine to L-aspartate with the release of ammonia. They were first identified in the blood plasma of guinea pigs by Clementi (1922) and later also found in bacteria and plants (Chibnall, 1930). Kidd (1953) reported the antitumour activity of the guinea pig serum against certain types of lymphomas and Broome (1961) related this effect with Clementi's observations and ascribed it to asparaginase activity. This supposition instigated intense investigations of asparaginases, including crystallographic studies. Only some L-asparaginases, those with high affinity towards the substrate ($K_M \simeq 10^{-5}\,M$), show antitumour activity. In bacteria, such high-affinity enzymes are found in the periplasm and are termed type II, in contrast to type I isoenzymes which are cytosolic and have lower substrate affinity. Type II enzymes isolated from *E. coli* (EcAII) and *Erwinia chrysanthemi* (ErA) are successful drugs in the treatment of acute lymphoblastic leukaemia, leukaemic lymphosarcoma and lymphosarcoma (Hill *et al.*, 1967).

The crystal structures of several type II bacterial asparaginases are known. They include the enzyme from *E. coli* with bound aspartate (Swain *et al.*, 1993; PDB accession code 3eca) and its active-site Thr89Val mutant with covalently bound product (Palm *et al.*, 1996), as well as enzymes from *Er. chrysanthemi* (ErA; Miller *et al.*, 1993), *Wolinella succinogenes* (WsA; Lubkowski *et al.*, 1996), *Acinetobacter glutaminasificans* (AGA; Lubkowski, Wlodawer, Housset *et al.*, 1994) and *Pseudomonas 7A* (PGA; Lubkowski, Wlodawer, Ammon *et al.*, 1994; Jakob *et al.*, 1997). The enzymes are homotetramers with nearly ideal 222 symmetry composed of four identical subunits (in EcAII, 326 amino-acids each) denoted *A*, *B*, *C* and *D*. The active site is created by subunits *A* and *C* or *B* and *D*. Therefore, the asparaginase tetramer can be treated as a dimer of dimers. The molecules of the reaction product, L-aspartate, found in the crystal structures of the enzymes define the location of the active site and the key surrounding residues Thr12, Tyr25, Ser58, Thr89, Asp90 and Lys162 (in the EcAII sequence). The role of these residues has been confirmed by mutagenesis and kinetic studies (Röhm & Van Etten, 1986; Bagert & Röhm, 1989; Derst *et al.*, 1992, 1994), leading eventually to a proposed mechanism of action that is similar to that of the reaction catalyzed by serine proteases (Rao *et al.*, 1996). The role of the Ser-His-Asp catalytic triad of serine proteases can be played in L-asparaginases by a similar triad, Thr89-Lys162-Asp90, which is conserved in the sequences and three-dimensional structures of all bacterial enzymes (Dodson & Wlodawer, 1998).

The Ser58Ala mutant of EcAII was prepared at the Institute of Physiological Chemistry, Philipps University, Marburg, by Professor K. H. Röhm. The mutated residue, Ser58, is involved in the native enzyme in binding of the reaction product in the active site through its side-chain OH group. The Ser58Ala mutant is therefore of interest because of its different substrate/product affinity (Wehner, 1993). The crystal structure of this mutant should explain the mechanism of substrate recognition.

Table 1
Summary of data collection.

	Crystal from agarose-gel crystallization, pH 4.8	Crystals from liquid-droplet crystallization, pH 9.0
Temperature (K)	295	120
Space group	$P2_12_12$	$P2_12_12$
Unit-cell dimensions (Å)		
a	226.9	222.3
b	128.3	125.1
c	61.9	60.9
Total no. of reflections	559228	354923
No. of unique reflections	66775	48151
Completeness (%)	85.4	90.5
Resolution (Å)	2.33	2.6

2. Crystallization

A new crystal form of *E. coli* L-asparaginase II (Ser58Ala mutant) has been obtained using two different crystallization conditions. Single crystals suitable for diffraction experiments could be grown using a modification of the conditions reported originally for native EcAII (Swain *et al.*, 1993) and under entirely new conditions established by the sparse-matrix method (Jancarik & Kim, 1991) using the Crystal Screen II kit (Hampton Research, California, USA). All crystallization experiments were conducted at 293 K using the vapour-diffusion method and the hanging- or sitting-drop technique (McPherson, 1982). The protein concentration determined by UV absorption at 280 nm was 10 mg ml^{-1} in 10 m*M* sodium acetate buffer pH 4.8.

In the first crystallization experiment, based on the conditions reported by Swain *et al.* (1993), MPD alone rather than mixed with PEG 3350 was used as the precipitating agent. Moreover, the crystals were grown in agarose gel (as described by Robert *et al.*, 1992) rather then in liquid droplets. 5 µl protein samples were mixed on siliconized cover slips with equal amounts of lukewarm (303 K) solution containing 100 m*M* sodium acetate pH 4.8, 30% MPD, 2% agarose. The gellified droplets were equilibrated against 1 ml reservoir solution containing 100 m*M* sodium acetate pH 4.8, 35% MPD. Wedge-like crystals (Fig. 1*a*) appeared after 12 months and reached maximum dimensions of 3 × 0.4 × 0.2 mm within 18 months.

The other crystallization conditions were established using a screening kit. Sitting drops consisting of 3 µl protein solution and 3 µl reservoir solution were placed in Crystal Clear strips (Hampton Research) and equilibrated against 100 µl reservoir solution containing 30% PEG-MME 550, 100 m*M* Bicine pH 9.0, 100 m*M* NaCl. Crystals (Fig. 1*b*) appeared after 24 h and reached maximum dimensions of 0.2 × 0.3 × 0.4 mm after an additional 2 d.

3. Diffraction experiments

Diffraction data for a crystal grown in agarose gel at acidic pH were collected at room temperature using a 300 mm image-plate scanner (MAR Research) and Cu $K\alpha$ radiation generated from an SRA2 rotating-anode generator (Siemens) operated at 45 kV and 112 mA. The crystal was mounted in thin-walled quartz capillary with a small amount of mother liquor and was rotated by 0.8° during each exposure.

The second data set, for crystals grown in liquid droplets at basic pH, was collected at 120 K using synchrotron radiation (Brookhaven National Laboratory, beamline X9B; λ = 0.98 Å) and a 345 mm MAR Research image-plate scanner. The crystal was mounted in a nylon fibre loop and flash-frozen in a nitrogen-gas stream (Teng, 1990). The crystal-to-detector distance was 200 mm and the oscillation range was 1.2°.

Indexing and integration of the images was performed in *DENZO* and scaling of the intensity data in *SCALEPACK* from the *HKL* program package (Otwinowski & Minor, 1997).

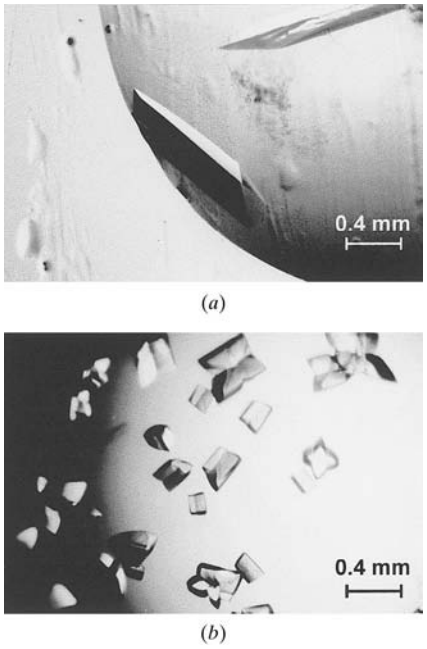

Figure 1
Single crystals of the Ser58Ala mutant of *E. coli* L-asparaginase II. (*a*) Crystals grown in agarose gel at acidic pH using MPD as precipitant. (*b*) Crystals grown at basic pH using PEG-MME 550 as precipitant.

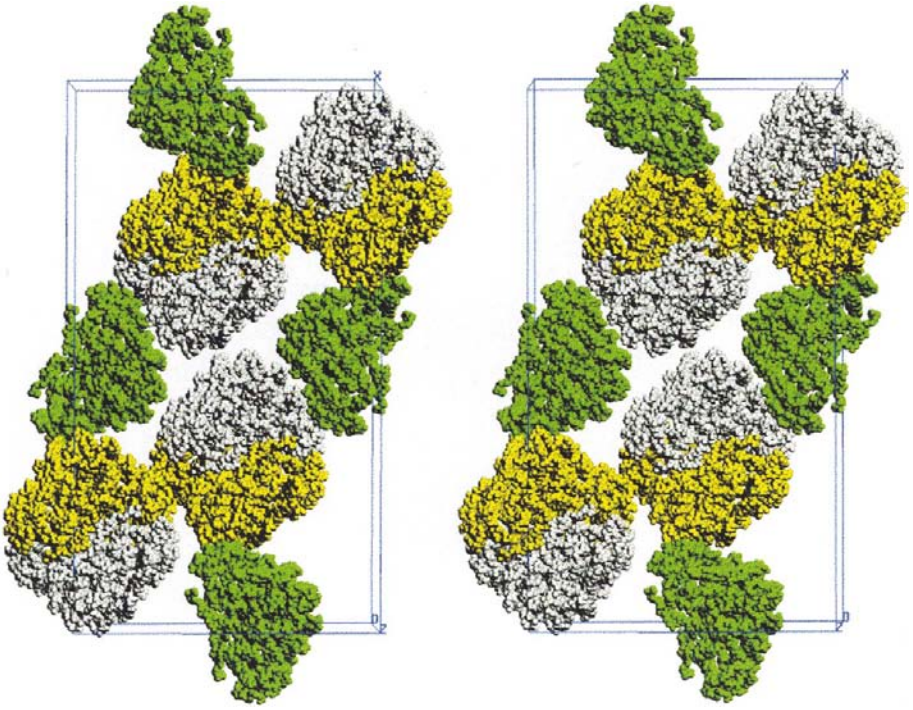

Figure 2
Stereoview of crystal packing of the new orthorhombic form of *E. coli* L-asparaginase II (Ser58Ala mutant). The dimers A^1C^1 (grey) and B^1D^1 (yellow) form a full homotetramer; from dimer A^2C^2 (green), the complete homotetramer is generated through the crystallographic dyad along [001]. This figure was drawn with *SETOR* (Evans, 1993).

4. Results

All crystals were very stable in the X-ray beam. A summary of crystal and experimental data is presented in Table 1.

The crystals grown from agarose gel at acidic pH diffracted X-rays to 2.33 Å resolution at room temperature. Merging of 559 228 reflections [with $I/\sigma(I) > 0.0$] resulted in a set of 66 775 unique reflections characterized by $R_{\rm int} = 0.092$ and $\langle I/\sigma(I)\rangle = 9.7$. The experimental data represented 85.4% of the theoretically possible reflections (65.0% in the last resolution shell 2.41–2.33 Å). The crystals belong to the $P2_12_12$ space group and have a unit cell characterized by the lattice parameters $a = 226.9$, $b = 128.3$, $c = 61.9$ Å.

For the crystals grown in liquid droplets at basic pH, 354 923 reflections with $I/\sigma(I) > 0.0$ were measured to 2.6 Å resolution in the low-temperature synchrotron-radiation experiment (with unit-cell parameters $a = 222.3$, $b = 125.1$, $c = 60.9$ Å). After merging, they were reduced to a unique data set consisting of 48 151 reflections. This data set is 90.5% complete (75.9% in the last resolution shell, 2.69–2.60 Å) and is characterized by $R_{\rm int} = 0.070$ and $\langle I/\sigma(I)\rangle = 16.6$.

The point symmetry of the tetramer precludes odd numbers of EcAII subunits in the asymmetric unit. An analysis of the Matthews volume (Matthews, 1968) for this new orthorhombic form of EcAII indicates that either four (3.07 Å^3 Da^{-1}) or six (2.05 Å^3 Da^{-1}) monomers could be accommodated in the asymmetric unit.

The crystal structure has been solved by molecular replacement using the *AMoRe* program package (Navaza, 1994) including data in the resolution range 15–3.5 Å. The active-site competent AC dimer of native EcAII (Swain *et al.*, 1993) served as a molecular probe. Three clear solutions for both the rotation and translation functions were obtained with a final R factor and correlation coefficient of 0.388 and 0.595, respectively. Rigid-body refinement of this solution decreased the R factor to 0.298 and improved the correlation coefficient to 0.751. This new orthorhombic form of EcAII contains one full homotetramer and one dimer in the asymmetric unit. The dimer is of AC type, *i.e.* it has all the components necessary for the formation of the active site. It is located close to the crystallographic dyad along [001] through which the complete homotetramer is generated. The crystal packing is shown in Fig. 2.

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